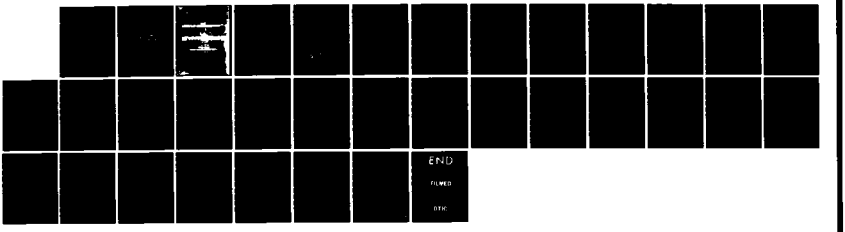
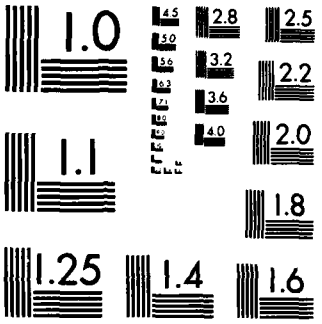
AD-A155 119 INTERACTION OF OPIATE AND PHENCYCLIDINE DERIVATIVES 1/1
WITH THE ACETYLCHOLINE RECEPTOR(U) NEW YORK STATE COLL
OF VETERINARY MEDICINE ITHACA R E OSWALD MAR 85
UNCLASSIFIED CRDC-CR-85004 DAAK11-83-C-0049 F/G 6/15 NL





(2)

CRDC-CR-85004

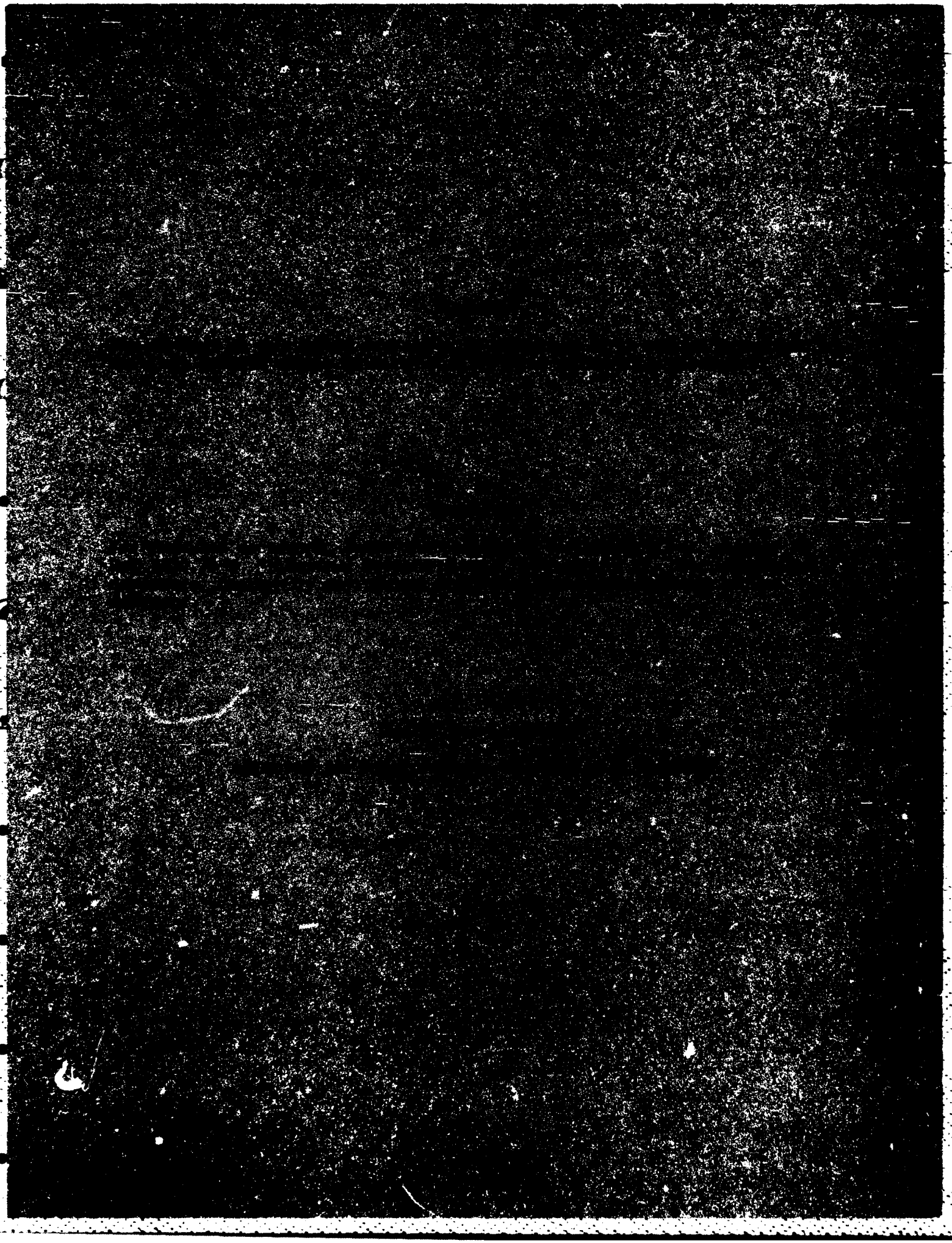
INTERACTION OF OPIATE AND PHENCYCLIDINE DERIVATIVES WITH THE ACETYLCHOLINE RECEPTOR

by Robert E. Oswald, Ph.D.

NEW YORK STATE COLLEGE OF VETERINARY MEDICINE
Ithaca, New York 14853-6401

March 1985

US Army Armament, Munitions & Chemical Command
Aberdeen Proving Ground, Maryland 21010-6423



UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) CRDC-CR-85004	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION New York State College of Veterinary Medicine 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Ithaca, NY 14853-6401	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION CRDC 8b. OFFICE SYMBOL (If applicable) SMCCR-RSP-C	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAAK11-83-C-0049
8c. ADDRESS (City, State, and ZIP Code) Aberdeen Proving Ground, MD 21010-5423	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO. / WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)
Interaction of Opiate and Phencyclidine Derivatives with the Acetylcholine Receptor

12. PERSONAL AUTHOR(S)
Oswald, Robert E., Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Contractor	FROM 83/6 TO 84/10	1985, March	39

16. SUPPLEMENTARY NOTATION

COR: William P. Ashman, SMCCR-RSP-C, (301) 671-3518

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
15	02	
06	15	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Acetylcholine Receptor, Phencyclidine, Cholinergic Receptor, Neuromuscular Junction, Opiates,

19. ABSTRACT (Continue on reverse if necessary and identify by block number)
The purpose of this work was to define the structural and biochemical environment of the AChR/I and the relationship of drug structure to the resultant pharmacological response. A number of drugs were tested using both biochemical and electrophysiological tests. For the most part, compounds consisted of benzomorphan and phencyclidine analogs, with the benzomorphans being the more thoroughly tested. Several important portions of the benzomorphan structure were identified, and the results of varying substitutents suggested that hydrophobicity parameters were the most important determinants of binding affinity.

Partial Support Keywords Include:

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL BRENDA C. ECKSTEIN	22b. TELEPHONE (Include Area Code) (301) 671-2914 / 22c. OFFICE SYMBOL SMCCR-SPS-IR

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

PREFACE

The work in this report was authorized under Contract No. DAAK11-83-0049. Work was started in June 1983 and was completed in October 1984.

The use of trade names in this report does not constitute an official endorsement or approval of the use of such commercial hardware or software. This report may not be cited for purposes of advertisement.

This report has been approved for release to the public.

Acknowledgments

I would like to thank Prof. Alexander Gero and Dr. A. E. Jacobson for providing compounds used in these studies, M. J. Bamberger for helpful discussions, and J. Bigelow and L. Michel for excellent technical assistance.

CONTENTS

		Page
1.	INTRODUCTION	5
2.	SUMMARY OF AChR/I STRUCTURE AND BINDING SITES	7
2.1	Agonist Sites	8
2.2	Noncompetitive Blocker Site	8
2.3	Opiate Site	9
2.4	Lipid-Associated Sites	9
3.	APPROACHES	9
3.1	Biochemical Methods for Analysis of Torpedo AChR	9
3.1.1	Preparation of AChR-Rich Membrane Fragments	9
3.1.2	Test for Interaction with the ACh Binding Site	10
3.1.3	Test for Interaction with the High-Affinity Site for Noncompetitive Blockers	10
3.1.4	Test for an Interaction with the Opiate Site	11
3.2	Single-Channel Recording Methods	11
3.2.1	Inside-Out Patch	12
3.2.2	Outside-Out Patch	12
4.	RESULTS OF BIOCHEMICAL STUDIES	12
4.1	Structural Aspects of Benzomorphans	12
4.1.1	Position 0	12
4.1.2	Position 1	13
4.1.3	Position 2	13
4.1.4	Position 3	13
4.1.5	Position 4	13
4.1.6	General Conclusions	13
4.2	Results Using Other Compounds	13
4.2.1	Other Opiate Derivatives	13
4.2.2	Phencyclidine Analogs	14
5.	RESULTS OF ELECTROPHYSIOLOGICAL STUDIES	14
5.1	(-)[^{3}H]ANMC	15
5.2	[^{3}H]PCP	15
5.3	Tiletamine	16
5.4	Summary	16
6.	LITERATURE CITED	17
APPENDICES:	A. Tables	18
	B. Figures	29
	C. Overall Status of the Report	37

INTERACTION OF OPIATE AND PHENCYCLIDINE DERIVATIVES WITH THE ACETYLCHOLINE RECEPTOR

1. INTRODUCTION

The purpose of the work performed under the present contract was to define the structural and biochemical environment of the AChR/I and the relationship of drug structure to the resultant pharmacological response. A number of drugs were tested using both biochemical and electrophysiological tests. For the most part, compounds consisted of benzomorphan and phencyclidine analogs, with the benzomorphans being the more thoroughly tested. Several important portions of the benzomorphan structure were identified, and the results of varying substituents suggested that hydrophobicity parameters were the most important determinants of binding affinity.

2. SUMMARY OF AChR/I STRUCTURE AND BINDING SITES

A wide variety of drugs and toxins interact with the cholinergic system of nerve and muscle cells. The major sites of action of these compounds include: (1) the synthesis and release mechanisms for acetylcholine (ACh) at the presynaptic nerve terminal, (2) the acetylcholine receptor (AChR) on the postsynaptic membrane, and (3) acetylcholinesterase (AChE). We have been studying the sites of interaction of compounds with the nicotinic AChR found on skeletal muscle and in electroplaque tissue (a tissue embryonically related to muscle). A number of distinct binding sites on the AChR have been defined and partially characterized (Heidmann et al., 1983; Oswald et al., 1984a&b; Albuquerque et al., 1980). These include:

a. Agonist Sites. The binding of ACh and other agonists to these sites (two per AChR molecule) results in the opening of the ion channel associated with the receptor molecule. Competitive antagonists, such as the plant toxin d-tubocurarine, also bind to these sites.

b. Noncompetitive Blocker Site. This site exists in one copy per AChR molecule and binds such molecules as local anesthetics, phencyclidine (PCP), and histrionicotoxin. This site may be located in the ion channel of the AChR and seems to block function by both a direct plugging of the channel and an increase in receptor desensitization.

c. Opiate Site. This site (one per AChR monomer) binds a class of opiate drugs, the benzomorphans. Binding to this site results in an allosteric activation of the AChR characterized by an increased mean channel open time in response to agonist.

d. Lipid-Associated Site. A finite number of lipid-associated sites (10 to 40 per AChR) are capable of blocking AChR function by enhancing desensitization.

The major goal of these studies was to define the structural aspects of drugs and toxins which confer the ability to interact with the first three sites.

Membrane fragments can be purified in decigram quantities from the electric organ of the ray Torpedo californica in which 50% of the protein content is AChR. Following detergent solubilization and purification, an AChR

preparation consists of four tightly associated polypeptide chains of 40 000 (α), 50 000 (β), 60 000 (γ), and 66 000 (δ) daltons present in a ratio of 2:1:1:1 (Reynolds and Karlin, 1978; Raftery, 1980; Lindstrom et al., 1979). The skeletal muscle AChR has an identical subunit composition. The sequence of all four subunits has been inferred from cDNA sequences (Noda et al., 1983).

The α subunit can be labeled by affinity reagents which are analogs of ACh and, therefore, carries at least a portion of ACh binding site (Karlin, 1980). A photoaffinity derivative of a noncompetitive blocker, 5-azido[^{3}H]-trimethisoquin (Waksman et al., 1980) was shown to label the δ subunit of the AChR with a specificity similar to that of the reversible binding of [^{3}H]PCP (Oswald et al., 1980; Oswald and Changeux, 1981b). That is, the labeling is enhanced by carbamylcholine and blocked by other noncompetitive blockers. In addition, many noncompetitive blockers can be cross-linked covalently to the AChR simply by irradiation of the AChR-noncompetitive blocker complex (Oswald and Changeux, 1981a) with ultraviolet light (254 nm). In most cases the results supported the notion that the δ chain carries a major portion of the site for noncompetitive blockers. One exception was [^{3}H]chlorpromazine which labeled all four types of AChR subunits with the specificity of the high-affinity site for noncompetitive blockers (Oswald and Changeux, 1981a). More detailed studies (Heidmann, Oswald, and Changeux, 1983) have demonstrated that the labeling of the four subunits is a result of binding to one high-affinity binding site on the AChR, corresponding to the binding site for PCP. This suggests that at least four, and possibly all five, subunits may contribute to a single common binding site. The high-affinity opiate binding site is associated with a portion of the δ subunit near the C-terminal (Oswald et al., 1984b). Peptide mapping experiments suggest that this site is physically distinct from the site for noncompetitive blockers.

Of the four classes of binding sites discussed earlier in this section only the first three are true binding sites on the AChR itself. The fourth, the lipid-associated sites, is probably mediated by partitioning into the membrane. The general properties of the sites are discussed below.

2.1 Agonist Sites.

Two high-affinity (K_D for acetylcholine ≅ 10 nM) binding sites exist on the AChR (rev. Karlin, 1980). These sites seem to mediate both channel activation and desensitization (rev. Changeux, 1981), although some controversy still exists (Dunn and Raftery, 1981). Other agonist sites of lower affinity have been proposed (Dunn and Raftery, 1981; Takeyasu et al., 1983; Oswald et al., 1984b) but have not been directly measured in a binding assay or characterized to any extent. Binding to the two high-affinity sites is blocked by the elapid neurotoxin, αbungarotoxin (αBgt), and the concentration of sites in a given preparation can be titrated precisely using α[^{125}I]Bgt.

2.2 Noncompetitive Blocker Site.

One high-affinity noncompetitive blocker site exists per AChR monomer and this site seems to be associated with the ion channel (Heidmann, Oswald and Changeux, 1983). Measurements of end-plate currents and single-channel records suggest that these agents are capable of shortening the mean channel lifetime (Neher and Steinbach, 1978) and increasing the rate of desensitization (Albuquerque et al., 1980). Binding studies indicate that these compounds bind

with higher affinity at equilibrium in the presence of cholinergic agonists (relative to no effectors) and that this increase in affinity is blocked by αBgt (Krodel et al., 1979; Eldefrawi et al., 1980; Oswald et al., 1983). Detailed kinetic studies of a representative noncompetitive blocker, [^{3}H]PCP, indicated that the increase in binding affinity was not due to binding to the desensitized conformation but rather to a conformation represented by only a small fraction of the total AChR population (Oswald et al., 1984a). Single-channel data with PCP (Oswald and Tank, in preparation) indicates that the EC50 for the shortening of the mean channel lifetime by PCP is identical to the microscopic affinity for the population to which [^{3}H]PCP binds, suggesting that the binding of [^{3}H]PCP in the presence of agonist is to the active, ion-conducting conformation. This notion is supported by the finding that the initial rate of [^{3}H]PCP binding is accelerated by at least 100-fold under conditions where a large proportion (> 90%) of the channels are in the active state (Eldefrawi et al., 1980; Oswald et al., 1983). Thus, in the case of the noncompetitive blocker, [^{3}H]PCP, in the presence of agonist, binding reflects an interaction with the active conformation.

2.3 Opiate Site.

The opiate binding site, reflected by the high-affinity (K_D = 0.2 μM) binding of the benzomorphan [^{3}H]N-allyl-N-normetazocine ([^{3}H]ANMC), exists in one copy per AChR monomer (Oswald et al., 1984b). Affinity labeling and peptide mapping studies as well as detailed competitive binding experiments (Oswald et al., 1984b) indicate that this binding site is physically distinct from the site for noncompetitive blockers. Preliminary single-channel recording data suggest that the mean channel lifetime is increased by these agents, and the binding of fluorescent agonist suggests that the percentage of desensitized AChR is decreased by these agents. In addition, the affinity of [^{3}H]ANMC binding is decreased by cholinergic agonists and antagonists. These data indicate that both the binding site for opiates and functional consequences of opiate binding differ from noncompetitive blockers. Preliminary kinetic analysis suggests that the high-affinity binding is to the resting conformation.

2.4 Lipid-Associated Sites.

The lipid-associated "sites" are associated with the AChR in the sense that a functional effect (i.e., increase in the percentage of desensitized AChR) can be observed and that the number of sites are finite (i.e., the binding of the radioactive compound is saturable) for a given protein-to-lipid ratio (Heidmann et al., 1983). The number of sites, however, varies with protein-to-lipid ratio. These sites can only be detected with a radioactive ligand or indirectly by measuring conformational transitions with a fluorescent agonist. The affinity of compounds for this site is independent of AChR conformation.

3. APPROACHES

This section includes a discussion of the techniques used to develop structure-activity relationships for the AChR and some of the advantages and limitations of these techniques.

3.1 Biochemical Methods for Analysis of Torpedo AChR.

3.1.1. Preparation of AChR-Rich Membrane Fragments.

The electric organs are dissected out and homogenized (1:1, w/w) in Buffer A (3 mM EDTA, 3 mM EGTA, 0.5 µg/ml pepstatin, 5 units/ml aprotinine, 0.05 M Tris-HCl, pH 7.5) using a Waring blender (2 periods of 1 min at full speed). The homogenate is centrifuged at 5000 rpm for 10 min (Beckman JA10 rotor); the pellet is recovered, rehomogenized, and recentrifuged (5000 rpm for 10 min). The two supernatants are combined and centrifuged at 7000 rpm for 2 hr (Beckman JA10 rotor). The pellet is resuspended in Buffer A and adjusted to 34% sucrose (w/w) in Buffer A; 40 mls are layered on a 25-ml cushion of 43% (w/w) sucrose in Buffer A. The tubes are then centrifuged at 40,000 rpm for 2.5 hr (Beckman Ti45 rotor). The interface between the two sucrose layers, containing AChR-rich membranes, is collected, diluted 3-fold with Buffer A, and centrifuged at 40 000 rpm for 45 min (Beckman Ti45 rotor). The pellet is resuspended in 20% (w/w) sucrose and layered on a 30 to 43% (w/w) sucrose gradient. The gradient is centrifuged at 22,000 rpm for 12 hr (Beckman SW27 rotor); and 2-ml fractions are collected. The peak containing AChR-rich membranes, identified by its ability to bind α[^{125}I]Bgt, is pooled. The membranes are stored in liquid nitrogen until use. Before use, membranes are centrifuged and resuspended in the appropriate volume of 50 mM 3-[N-morpholino]propanesulfonic acid (MOPS)-NaOH, pH7.5, 1 mM EGTA). These membranes contain the four receptor subunits as well as a number of nonreceptor peptides. The major nonreceptor peptides are a 43,000 dalton chain, which may be a structural protein associated with the AChR, and a number of peptides in the 95,000 dalton region which probably represent both a Na^+/K^+ ATPase and an anion channel.

3.1.2 Test for Interaction with the ACh Binding Site.

The analysis of the interaction of a compound with the ACh binding site was investigated using the binding of α[^{125}I]bungarotoxin (α[^{125}I]Bgt). α[^{125}I]Bgt binding assays provide a rapid, inexpensive, and convenient measure of the ability of a compound to bind to the ACh binding site. Also, because of the relative insensitivity of α[^{125}I]Bgt binding to conformational states of the AChR, only competitive inhibition is generally detected. The procedure consists of incubating membrane fragments (10 nM in αBgt sites) with the compound to be tested (10 to 30 min) and then adding 1 nM α[^{125}I]Bgt (total volume of 0.7 ml). The initial rate of α[^{125}I]Bgt binding is then measured by filtering aliquots (0.05 ml) through ECWP millipore filters (presoaked in 1 mg/ml bovine serum albumin and 1 µM αBgt) and washing the filters three times with MOPS-NaOH buffer. An estimate of the affinity of the compound can then be made by calculating the "protection constant," K_p, which is given by Weber & Changeux (1974):

$$V_i = V_{i,o} \frac{K_p}{K_p + F}$$

Where $V_{i,o}$ is the initial rate measured in the absence of the compound and V_i is the initial rate measured in the presence of a given concentration, F, of the compound.

3.1.3 Test for Interaction with the High-Affinity Site for Noncompetitive Blockers

The high-affinity site for noncompetitive blockers on the Torpedo AChR is analyzed using [^{3}H]PCP as a radioactive probe. [^{3}H]PCP binding to

the AChR is measured most accurately and rapidly using a filtration assay. [^{3}H]PCP (50 nM in initial experiments) is incubated with AChR-rich membranes (0.1 μM in αBgt sites) in the presence or absence of 0.2 mM carbamylcholine for 30 min in MOPS/NaOH/EGTA buffer. The reaction is terminated by filtration of a 0.1-ml aliquot through a Whatman GF/B filter and washing rapidly with 3 ml of buffer. Radioactivity is assayed by scintillation counting of the filter. All measurements are made in the presence or absence of carbamylcholine (an AChR agonist). This is done because agonists (and some antagonists), are capable of regulating the binding of [^{3}H]PCP to the AChR. That is, the affinity of the AChR for [^{3}H]PCP is 5- to 10-fold higher in the presence than in the absence of agonists. A compound could have the effect of either increasing allosterically the affinity or decreasing competitively or allosterically the affinity of the AChR for [^{3}H]PCP.

3.1.4 Test for an Interaction with the Opiate Site.

The high-affinity binding site for opiates can be analyzed effectively with the benzomorphan, [^{3}H]ANMC. Binding is measured with a filtration assay identical to that for [^{3}H]PCP except that 50 nM [^{3}H]ANMC is used instead of [^{3}H]PCP. Competition experiments are performed at constant [^{3}H]ANMC and AChR concentrations with the concentration of test compound varying over approximately four orders of magnitude.

3.2 Single-Channel Recording Methods.

Primary rat skeletal muscle cultures were used. This preparation consists of obtaining myoblasts by mincing the leg muscles of 18-day-old fetal rats. The minced tissue is then digested for 1 hr in 0.025% pancreatin in DMEM, dissociated further by pipetting, and plated on tissue culture dishes in 10% fetal calf serum in DMEM. Cells are treated with cytocine arabinosine to prevent overgrowth of fibroblasts. The myoblasts are allowed to fuse to form myotubes. Myotubes contain a mixture of AChRs with junctional and extrajunctional characteristics, with the extrajunctional AChRs predominating. The medium is removed immediately before the experiment and replaced with a Ringer's solution (120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES-NaOH, pH 7.4).

The recording configuration is essentially that described by Hamill et al. (1981). A Sylgard-coated, fire-polished micropipette (borosilicate glass; W. P. Instruments, TW 150-6) is held in a microelectrode holder (W. P. Instruments MEH-2R) driven by a MO-102 Narishige micromanipulator. Manipulations and preparations are viewed using phase-contrast or Nomarski optics with a Nikon Diaphot inverted microscope at 400X magnification. The Dagan patch clamp with a 10 GΩ probe is used. Single-channel records are stored on FM tape. Data is digitized (AR11 10 bit A/D converter) at 50 to 100 μsec intervals in 400K byte segments and stored on an RL02 disk. The records are then analyzed using a Digital PDP 11/24 computer.

For measurements of single-channel conductances and single-channel lifetimes, the pipette is mounted in the recording apparatus and positive pressure is applied to the back of the pipette. The pipette is then lowered until the tip contacts the cell. Upon contact with the cell, a gentle suction is applied which results in the formation of a 20 to 100 GΩ seal. Two recording configurations are used:

3.2.1 Inside-Out Patch.

In this case, the patch of membrane is allowed to remain attached to the cell and the compound to be tested (with or without 0.1 μM ACh) remains in the pipette. A typical high-sodium Ringer's solution is used. This recording configuration is easy to obtain (with the success rate of forming seals on the order of 70 to 80%) but does not allow changes of solution.

3.2.2 Outside-Out Patch.

In this case, the pipette is filled with 150 mM KCl and 3 mM HEPES-NaOH, pH 7.4, without agonist or the compound to be tested. The gigaseal is formed, and a negative pressure is applied until the seal becomes "leaky." At this point, the pipette is withdrawn from the cell. The gigaseal forms with the extracellular face of the membrane facing out. At this point, agonist and/or the compound to be tested can be applied to the outside tip of the pipette using a flow system in which the pipette is placed at the orifice of a tube from which a buffer containing agonist and/or the compound to be tested is flowing. The use of several of these tubes allows rapid changes of solutions. Thus, the advantage of this procedure is that several concentrations of a compound can be tested on the same preparation.

4. RESULTS OF BIOCHEMICAL STUDIES

This section includes a discussion of the results of experiments in which compounds were tested for their ability to inhibit [^{3}H]PCP, [^{3}H]ANMC, and α[^{125}I]Bgt binding. The first portion of the discussion considers the effect of varying substituents on five sites of the benzomorphan backbone. The second portion is a more general discussion of the compounds tested. The third section discusses the relationship between the PCP site and the (-)ANMC site.

4.1 Structural Aspects of Benzomorphans.

Figure 1 in appendix B shows the structure of the benzomorphan backbone and the nomenclature used. Tables 1 through 5 in appendix A catalog the results of this study. The following discussion is organized according to the site on the benzomorphan backbone:

4.1.1 Position 0.

The largest number of analogs was available for this portion of the study, and the results are shown in Table 1. The inhibition of [^{3}H]ANMC and α[^{125}I]Bgt binding by these analogs varies somewhat, but consistent trends are not evident. On the other hand, the inhibition of [^{3}H]PCP binding, particularly in the presence of carbamylcholine, shows a consistent trend with variations in the R_0 position. The K_i for a given analog decreases with increasing hydrophobicity of the side chain, so that pentazocine (AJ4), the most hydrophobic, is 10-fold more potent than the hydrogen analog (G4). The hydrophobicity (measured on a benzene ring; Hansch, 1983) varies in the following order:

$CH_2CH_2\emptyset$ > $CH_2{=}C(CH_3)_2$ > or ≈ CH_2  > or ≈ $CH_2CH{=}CH_2$ > CH_3 > H.

This is the order seen for the inhibition of [^{3}H]PCP binding in the presence of carbamylcholine. This parameter is, of course, interrelated with steric factors; however, one would expect steric hindrance to follow the opposite rank order.

4.1.2 Position 1.

The R_1 group is on the aromatic ring, so that one might expect that the electronic, as well as hydrophobic, parameters may be important. Comparisons among a hydroxyl, a methoxy, and a hydrogen and among a hydroxyl, a fluorine, and a hydrogen are given in Table 2. Again, the major variations seem to be for [^{3}H]PCP in the presence of carbamylcholine. In this case, the major difference is in the substitution of a hydroxyl group which seems to decrease activity relative to fluorine, methoxy, and hydrogen. This is consistent with a hydrophobicity effect (decreased affinity with decreased hydrophobicity), rather than an electronic effect.

4.1.3 Position 2.

The number of systemically varying derivatives in this position is smaller, but the results are consistent with an increased affinity with increased hydrophobicity for both [^{3}H]PCP binding and for [^{3}H]ANMC binding (Table 3).

4.1.4 Position 3.

With a small number of analogs, the trend is for increased affinity with increased hydrophobicity for [^{3}H]PCP but not for [^{3}H]ANMC (Table 4).

4.1.5 Position 4.

Again, the trend in this position is for a decreased affinity with the addition of a hydroxyl group, which is consistent with a decreased affinity with decreased hydrophobicity (Table 5).

4.1.6 General Conclusions.

The general conclusions from these studies is that hydrophobicity is a major determinant for the inhibition of [^{3}H]PCP binding in the presence of carbamylcholine. This is true for all five sites and is consistent with a previous finding (Oswald et al., 1984a) that the driving force for the binding of [^{3}H]PCP is largely an increase in entropy. On the other hand, hydrophobicity seems to be a minor determinant for the inhibition of [^{3}H]ANMC and α[^{125}I]Bgt. In these cases, the major differences between the substituents are dictated by the stereospecificity, with (-) isomers being more potent in the case of [^{3}H]ANMC binding and (+) isomers being more potent in the case of α[^{125}I]Bgt binding.

4. Results Using Other Compounds.

Several other compounds were tested and fell into two classes: 1) other opiate derivatives and 2) phencyclidine derivatives.

4.2.1 Other Opiate Derivatives.

Several opiate derivatives other than those described in the previous section were tested and the results are summarized in Table 6. The addition of

APPENDIX B
FIGURES

Table 7
Phencyclidine Derivatives

		PCP(+CC)	PCP(-CC)	ANMC	Bgt
PCMP	(-)	0.07	0.72	0.4	
	(+)	0.08	0.89	1.9	
beta-DL-Dioxadrol		1.1	3.6	7.7	40.
beta-D-Dexoxadrol		0.57	3.3	5.8	84.
Tiletamine		7.1	5.6	19.	130.

Table 6
Other Opiate Derivatives

		PCP(+CC)	PCP(-CC)	ANMC	Bgt
Thebaine	(-)	63	436	56	61
	(+)	251	>1000	89	104
Oxymorphone	(-)	93	388	143	75
	(+)	>1000	>1000	615	112
5-m-hydroxyphenyl-2-methylmorphan	(-)	42	13	31	12
	(+)	44	9	61	27

Table 5
Position 4 Comparisons

	R_0	R_1	R_2	R_3	R_4	PCP(+CC)	PCP(-CC)	ANMC	Bgt
(R)04	CH_3	OH	CH_3	CH_3	H	33.2	17.0	13.8	229.
05	CH_3	OH	CH_3	CH_3	OH	75.9	34.3	22.9	1230.

Table 4
Position 3 Comparisons

	R_0	R_1	R_2	R_3	R_4	PCP(+CC)	PCP(-CC)	ANMC	Bgt
(-)09	CH_3	OH	CH_3	H	H	91.	155.	1.7	50.
04	CH_3	OH	CH_3	CH_3	H	34.	4.8	3.2	381.
(+)09	CH_3	OH	CH_3	H	H	158.	375.	24.	91.
04	CH_3	OH	CH_3	CH_3	H	32.	29.	25.	76.

Table 3
Position 2 Comparisons

	R_0	R_1	R_2	R_3	R_4	PCP(+CC)	PCP(-CC)	ANMC	Bgt
Bgt									
(-)09	CH_3	OH	CH_3	H	H	91.	155.	1.7	50.
010	CH_3	OH	C_2H_5	H	H	39.	208.	0.65	38.
(+)09	CH_3	OH	CH_3	H	H	158.	375.	24.	91.
010	CH_3	OH	C_2H_5	H	H	34.	227.	10.6	70.
(R)G9	$CH_2CH=C(CH_3)_2$	OH	CH_3	CH_3	H	12.7	8.5	5.7	107.
03	$CH_2CH=C(CH_3)_2$	OH	C_2H_5	CH_3	H	1.7	3.3	1.1	43.

Table 2
Position 1 Comparisons

	R_0	R_1	R_2	R_3	R_4	PCP(+CC)	PCP(-CC)	ANMC	Bgt
(R)05	CH_3	OH	CH_3	CH_3	OH	75.9	34.3	22.9	1230.
06	CH_3	H	CH_3	CH_3	OH	10.4	14.0	6.1	761.
07	CH_3	OCH_3	CH_3	CH_3	OH	9.5	12.6	47.9	129.
(R)04	CH_3	OH	CH_3	CH_3	H	33.2	17.0	13.8	229.
AJ9	CH_3	H	CH_3	CH_3	H	3.7	11.4	5.1	125.
AJ11	CH_3	F	CH_3	CH_3	G	2.2	4.4	4.7	60.

TABLE 1

Position 0 Comparisons

	R_0	R_1	R_2	R_3	R_4	PCP(+CC)	PCP(-CC)	ANMC	Bgt
(-)01	$CH_2CH{=}CH_2$	OH	CH_3	CH_3	H	6.6	4.6	0.72	405.
02	CH_2-△	OH	CH_3	CH_3	H	6.9	1.9	2.6	225.
03	$CH_2CH{=}C(CH_3)_2$	OH	CH_3	CH_3	H	7.0	2.0	2.0	117.
AJ4	$CH_2CH_2Ø$	OH	CH_3	CH_3	H	1.7	3.1	1.5	
04	CH_3	OH	CH_3	CH_3	H	34.4	4.8	3.2	381.
(+)01	$CH_2CH{=}CH_2$	OH	CH_3	CH_3	H	16.9	13.4	1.8	201.
02	CH_2-△	OH	CH_3	CH_3	H	9.5	26.7	9.2	218.
03	$CH_2CH{=}C(CH_3)_2$	OH	CH_3	CH_3	H	18.3	15.0	9.4	96.
04		OH	CH_3	CH_3	H	31.7	29.2	24.5	76.
(R)01	$CH_2CH{=}CH_2$	OH	CH_3	CH_3	H	11.9	9.0	1.3	303.
07	CH_2-△	OH	CH_3	CH_3	H	3.9	2.2	1.3	266.
03	$CH_2CH{=}C(CH_3)_2$	OH	CH_3	CH_3	H	12.7	8.5	5.7	107.
AJ4	$CH_2CH_2Ø$	OH	CH_3	CH_3	H	3.2	1.3	7.5	9.4
02	CH_3	OH	CH_3	CH_3	H	33.3	42.9	2.5	258.
05	H	OH	CH_3	CH_3	H	35.9	171.	0.39	103.

APPENDIX A
TABLES

BLANK

LITERATURE CITED

Albuquerque, E. X., Adler, M., Spivak, C. E., and Aguayo, L. (1980) Ann. N. Y. Acad. Sci., 358: 204-238.

Changeux, J. P. (1981) Harvey Lect. 75: 85-254.

Dunn, S. M. J. and Raftery, M. A. (1982) Proc. Natl. Acad. Sci. USA 79: 6757-6761.

Eldefrawi, M. E., Eldefrawi, A. T., Aronstam, R. S., Maleque, M., Warnick, J. E., and Albuquerque, E. X. (1980) Proc. Natl. Acad. Sci. USA, 77: 7458-7462.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) Pflügers Archiv. 391: 85-100.

Hansch, C. (1983) In: C. J. Cavallito (ed.) Structure-Activity Relationships, 1: 75-165, Pergamon, Oxford.

Heidmann, T., Oswald, R. E., and Changeux, J. P. (1983) Biochemistry, 22: 3112-3127.

Karlin, A. (1980) In: C. W. Cotman, G. Poste, and G. L. Nicholson (eds) The Cell Surface and Neuronal Function, pp. 191-269, Elsevier/North-Holland, Amsterdam.

Karpen, J. W., Aoshima, H., Abood, L. G. and Hess, G. P. (1982) Proc. Natl. Acad. Sci., USA, 79: 2509-2513.

Krodel, E. K., Beckman, R. A., and Cohen, J. B. (1979) Molec. Pharmacol. 15: 294-312.

Lindstrom, J., Merlie, J., and Yogeeswaran, G. (1979) Biochemistry, 18: 4465-4470.

Neher, E. and Steinbach, J. H. (1978) J. Physiol., Lond., 277: 153-176/

Noda, M., Takahashi, H., Tanabe, T., Toyasoto, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T., and Numa, S. (1983) Nature, 302: 528-532.

Oswald, R. E., Bamberger, M. J., and McLaughlin, J. T. (1984a) Molec. Pharmacol., 25: 360-368.

Oswald, R. E. and Changeux, J. P. (1981a) Biochemistry, 20: 7166-7174.

Oswald, R. E. and Changeux, J. P. (1981b) Proc. Natl. Acad. Sci., USA, 78: 3925-3929.

Oswald, R. E., Heidmann, T., and Changeux, J. P. (1983a) Biochemistry, 22: 3128-3136.

Oswald, R. E., Pennow, N. N., and McLaughlin, J. T. (1984b) Proc. Natl. Acad. Sci. USA, in press.

Oswald, R. E., Sobel, A., Waksman, G., Roques, B., and Changeux, J. P. (1980) FEBS Lett., III: 29-34.

Raftery, M. A., Hunkapiller, M. W., Strader, C. D., and Hood, L. E. (1980) Science 208: 1454-1457.

Reynolds, J. A. and Karlin, A. (1978) Biochemistry 17: 2035-2038.

Takeyasu, K., Udgaonkar, J. B. and Hess, G. P. (1983) Biochemistry 22: 5973-5978.

Waksman, G., Oswald, R., Changeux, J. P., and Roques, B. P. (1980) FEBS Lett. III: 23-28.

Weber, M. and Changeux, J. P. (1974) Molec. Pharmacol. 10: 15-34.

suggest that PCP may exhibit both a channel block mechanism and an increased equilibrium level of desensitization. This has been suggested by direct binding measurements (Oswald et al., 1984a), stopped flow measurements of fluorescent agonist binding (Heidmann et al., 1984), and quenched flow measurements of ion flux (Karpen et al., 1982).

5.3 Tiletamine.

As shown in Figure 5, tiletamine also decreased mean channel lifetime. Like PCP, this effect occurs at a concentration approximately tenfold lower than its apparent affinity for the PCP site. Also, very little voltage dependence was observed within the range tested. These results suggest that the mechanism of action of tiletamine is quite similar to that of PCP.

5.4 Summary.

The results presented here suggest two mechanisms of action for the compounds used in the present study. Compounds interacting with the ANMC may effect an allosteric increase in the mean channel lifetime. On the other hand, compounds interacting with the PCP site seem to decrease mean channel lifetime and increase the equilibrium level of desensitization.

conductance; however, the single-channel lifetime was modified as described below for the three drugs.

5.1 (-)ANMC.

The effects of (-)ANMC on single-channel lifetime was investigated with both cell-attached patches and outside-out patches in both a clonal muscle cell line (BC_3H-1) and primary cultures of rat myotubes. As shown in Figure 3, the mean channel lifetime of the BC_3H-1 AChR (cell-attached configuration) increases at low concentration of (-)ANMC and decreases at higher concentrations (ACh concentration of 100 nM). In two experiments with outside-out patches from rat myotubes, similar results were observed except that the channel lifetimes were longer due to the different cell type. These results suggest a possible correlation between the functional effects and the binding results. The high-affinity component observed in the binding studies may be an allosteric site capable of increasing the mean channel lifetime, and the low affinity component may be analogous to the site of channel blockade.

5.2 PCP.

For both outside-out and cell-attached configurations in rat myotubes, PCP decreased the mean channel lifetime with an IC50 on the order of 25 nM (Figure 4). This analysis was performed exclusively on the 50 pS AChR channel which predominates in these cells. An additional channel of 90 pS was also observed but comprised less than 1% of the total channel openings and was not analyzed further. In the outside-out configuration, ACh (100 nM) and varying concentration of PCP was applied to the outside of the pipette by a gravity-flow perfusion system, allowing the effects of different concentrations of PCP to be assessed on the same patch. The membrane potential was held at -125 mV (i.e., inside of the pipette negative with respect to the bath). In the case of the cell attached configuration, ACh and [^{3}H]PCP were added to the inside of the pipette, and a hyperpolarizing holding potential was adjusted so that the channels were 5 pA. This resulted in a constant driving force for the conditions tested. In addition to the decrease in mean open channel lifetime, the mean channel closed time increased and became more complex with an increase in PCP concentration (outside-out configuration). The mean channel closed time was fit reasonably well, in the absence of PCP, with a single exponential, although a much faster component (less than 1 msec) was probably present. PCP increased the mean closed time of the long component and increased the percentage of short closing. The absolute values varied between patch to patch. The voltage dependence of the blockade was studied using excised inside-out patches with [^{3}H]PCP and ACh in the pipette. No consistent difference was observed between holding potentials giving rise to a net inward driving force and these giving rise to a net outward driving force. This is consistent with the previous suggestion that the active species of PCP is the unprotonated (neutral) form (Oswald et al., 1984).

Several conclusions can be drawn: 1) PCP decreases the mean channel lifetime of the AChR at nanomolar concentrations. This is consistent with the data presented in Oswald et al. (1984a) which indicate that PCP binds to a conformation of the AChR kinetically similar to the open channel state. This conclusion of Oswald et al. (1984a), supported by this study, is that a portion of the AChR blockade by PCP may be due to a channel blockade; and 2) the increase in the mean closed time and increased percentage of rapid closing events

a ring and ether linkage in thebaine and oxymorphone or the change in the position of the aromatic ring in the methylmorphan dramatically decreased potency, but stereospecificity was maintained. For these compounds, as well as those of the previous section, the (-) isomer exhibited a decrease in the free energy of binding of 0.8 to 0.9 kcal/mole greater than the (+) isomer when the inhibition of [^{3}H]PCP or [^{3}H]ANMC binding was used as an indicator. Also tested were etonitazine and fentanyl derivates. Of potential interest, in these cases, is the relatively high affinity of the etonitazine derivative for the [^{3}H]PCP site in the presence of carbamylcholine. Because of the potential for irreversible binding, this compound may be a useful affinity label for the [^{3}H]PCP binding site.

4.2.2 Phencyclidine Analogs.

Several phencyclidine analogs were tested, and the results are summarized in Table 7. Again, when tested, some stereospecificity was observed. The most interesting compound tested was (-)PCMP, which exhibited an affinity of 60 nM for the [^{3}H]PCP site in the present of carbamylcholine. This is the highest affinity of any compound for either the [^{3}H]PCP or (-)ANMC site and may be a useful model reagent for further studies of structure-activity relationships.

4.3 Relationship Between (-)ANMC Site and PCP Site.

An additional question that arose in the course of these studies was whether or not the [^{3}H]ANMC binding site was distinct from the [^{3}H]PCP binding site. This question was addressed by performing binding curves for [^{3}H]-PCP (varying [^{3}H]PCP concentration with fixed AChR concentration) at various concentration of nonradioactive (-)ANMC and for (-)[^{3}H]ANMC at various concentration of nonradioactive PCP. As shown in Figure 2A, appendix B, the binding of (-)[^{3}H]ANMC in the absence of carbamylcholine is complex with at least two components (K_D's of 0.2 μM and 3 μM, each comprising approximately one binding site per AChR monomer). PCP inhibits the high-affinity site by a noncompetitive mechanism (apparent decrease in the total number of binding sites with no change in the K_D) but inhibits the low-affinity site by a competitive mechanism (apparent decrease in the K_D with no change in the number of binding sites). The K_I for both effects was approximately 7 μM. In the presence of carbamylcholine (Figure 2B), the binding of (-)[^{3}H]ANMC shows only one component with a K_D of 3 μM. The mechanism of inhibition in this case is difficult to characterize, but PCP decreased the binding with a K_I of approximately 0.2 μM. The binding of [^{3}H]PCP is comparatively simple, exhibiting a single component (one site per AChR monomer) with a higher affinity in the presence (K_D = 0.2 μM; Figure 2C) than in the absence (K_D = 2 μM; Figure 2D) of carbamylcholine. (-)[^{3}H]ANMC inhibits the binding competitively in both cases with a K_I of 6 μM. The simplest explanation of these data is that the high-affinity site for (-)[^{3}H]ANMC and the site for [^{3}H]PCP are distinct but allosterically coupled and that the low-affinity site for (-)[^{3}H]ANMC and the site for [^{3}H]PCP are identical.

5. RESULTS OF ELECTROPHYSIOLOGICAL STUDIES

A series of patch clamp experiments were performed in which the effects of (-)ANMC, PCP, and tiletamine on single-channel conductance and lifetime were investigated. In no case did a drug affect the single-channel

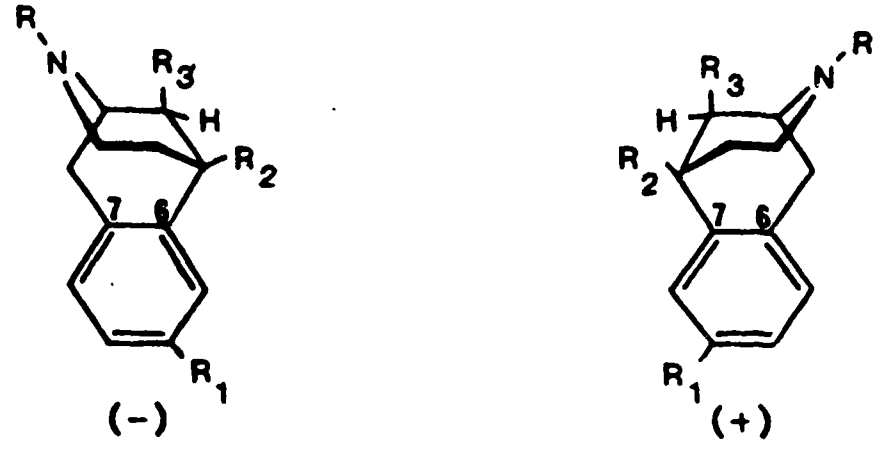


Figure 1. Structure of the benzomorphan backbone.

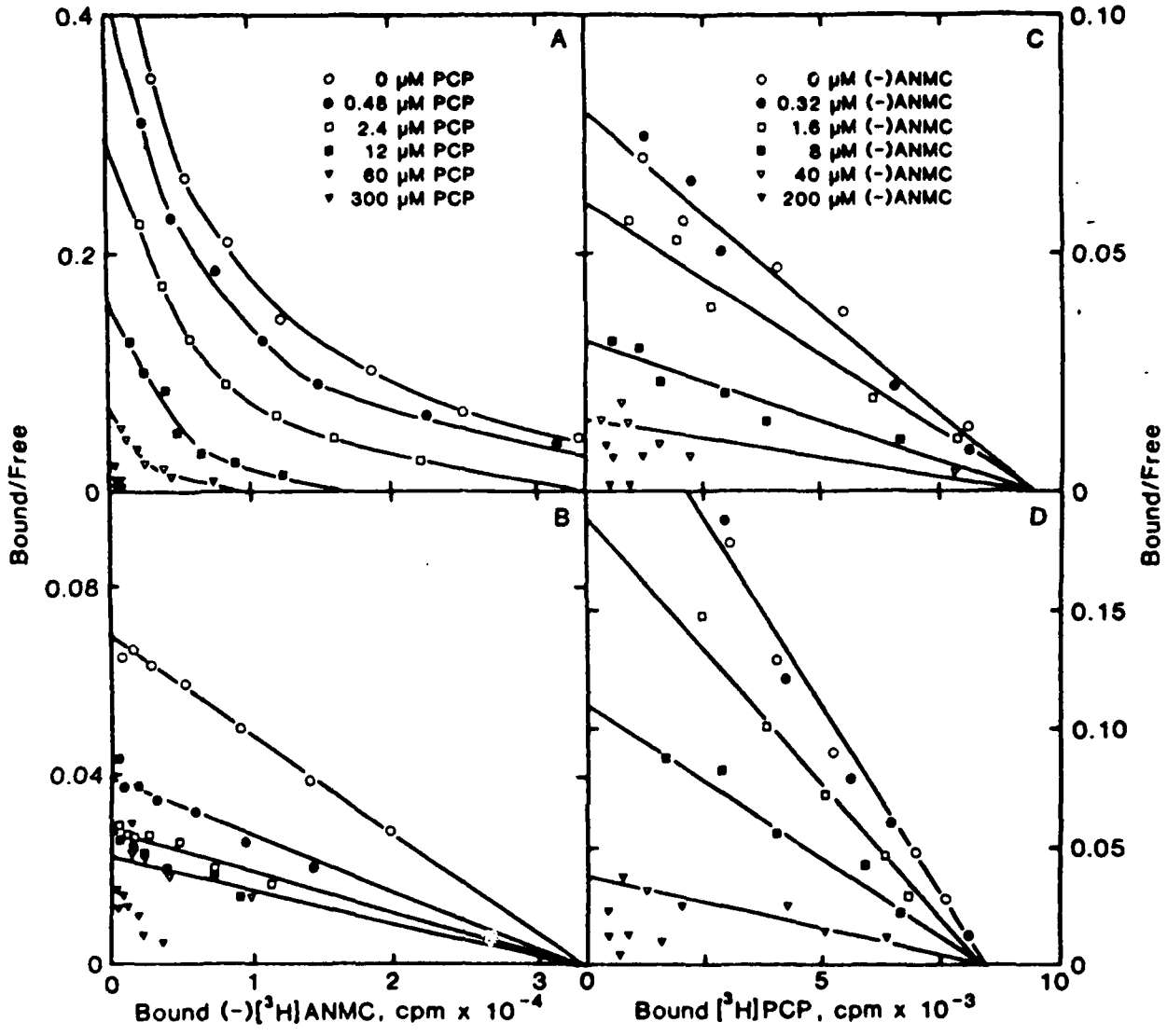


Figure 2. The binding of (-)[^{3}H]ANMC (A,B) and [^{3}H]PCP (C,D) to acetylcholine receptor-rich membrane fragments in the presence (B,D) or absence (A,C) of 0.2 mM carbamylcholine and varying concentrations of either nonradioactive PCP (A,B) or (-)ANMC (C,D). The data are plotted as Scatchard transformations of the "specific" binding determined following subtraction of "nonspecific" binding measured using either 0.2 mM nonradioactive (-)ANMC (A,B) or 0.2 mM nonradioactive PCP (C,D).

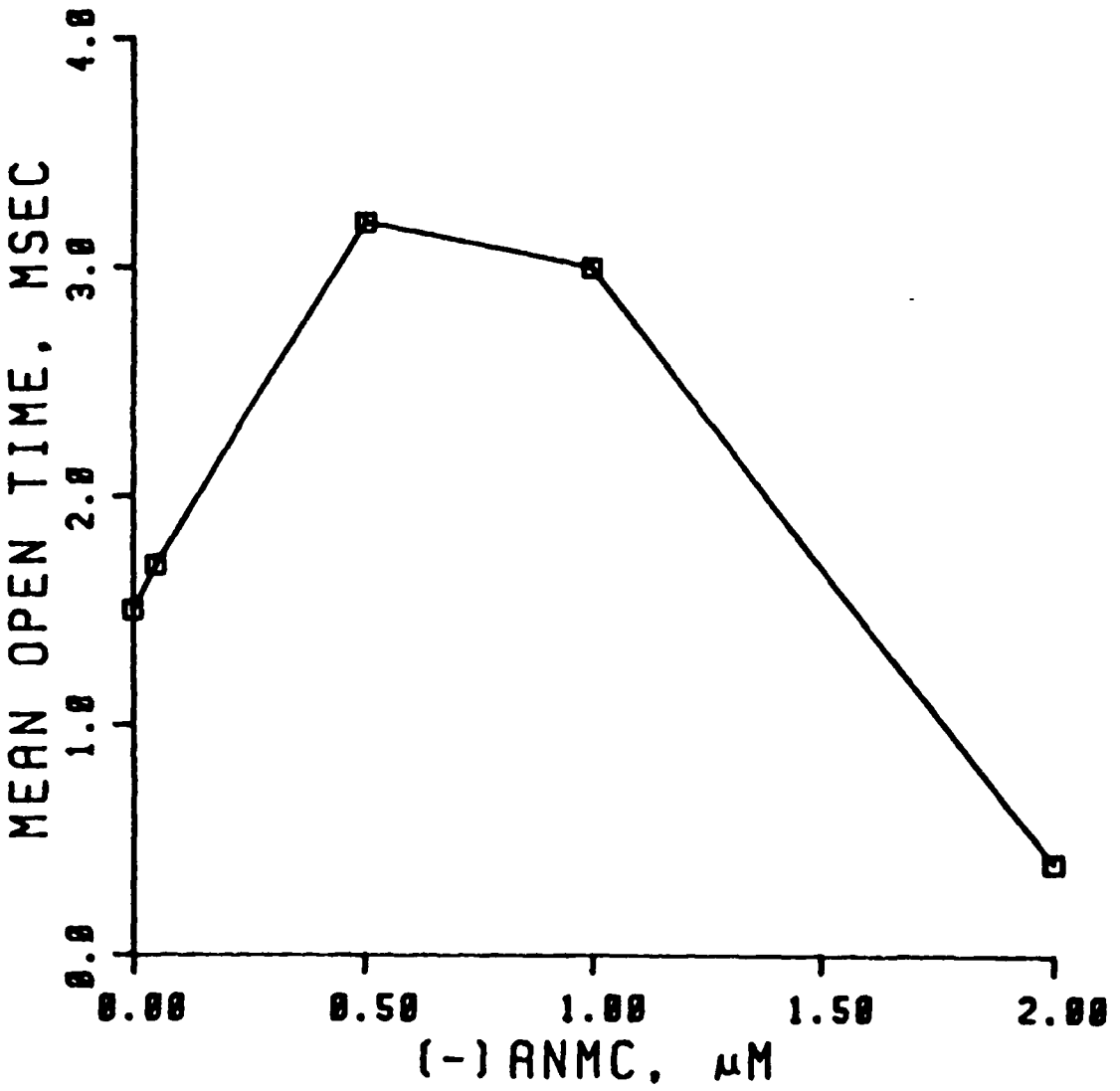


Figure 3. Effect of (-)ANMC on the mean channel open time of the acetylcholine receptor from BC3H1 cells. Acetylcholine (200 nM) and (-)ANMC were added to the inside of the pipette, and the cell-attached configuration was used. The membrane potential was adjusted to obtain a channel current of 5 pA.

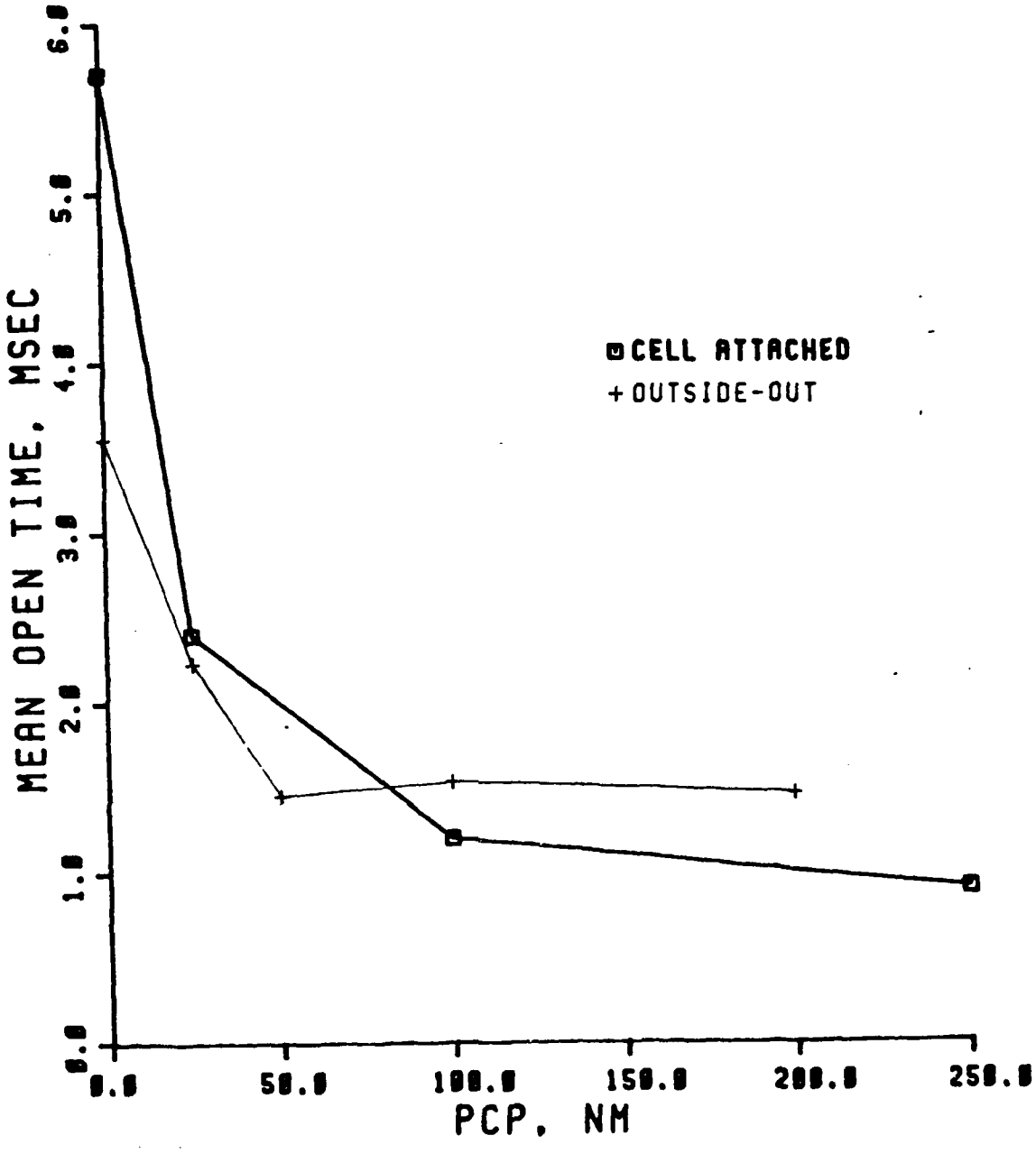


Figure 4. Effect of phencyclidine on the mean channel open time for the acetylcholine receptor from rat myotubes. Acetylcholine (100 nM) and phencyclidine were added to the pipette solution in the cell attached condition and were added to the outside of the pipette by a gravity-flow perfusion system in the outside-out configuration. In the outside-out condition, the inside of the pipette was held at -125 mV relative to the bath. In the cell attached condition, the membrane potential was adjusted to obtain a channel current of 5 pA.

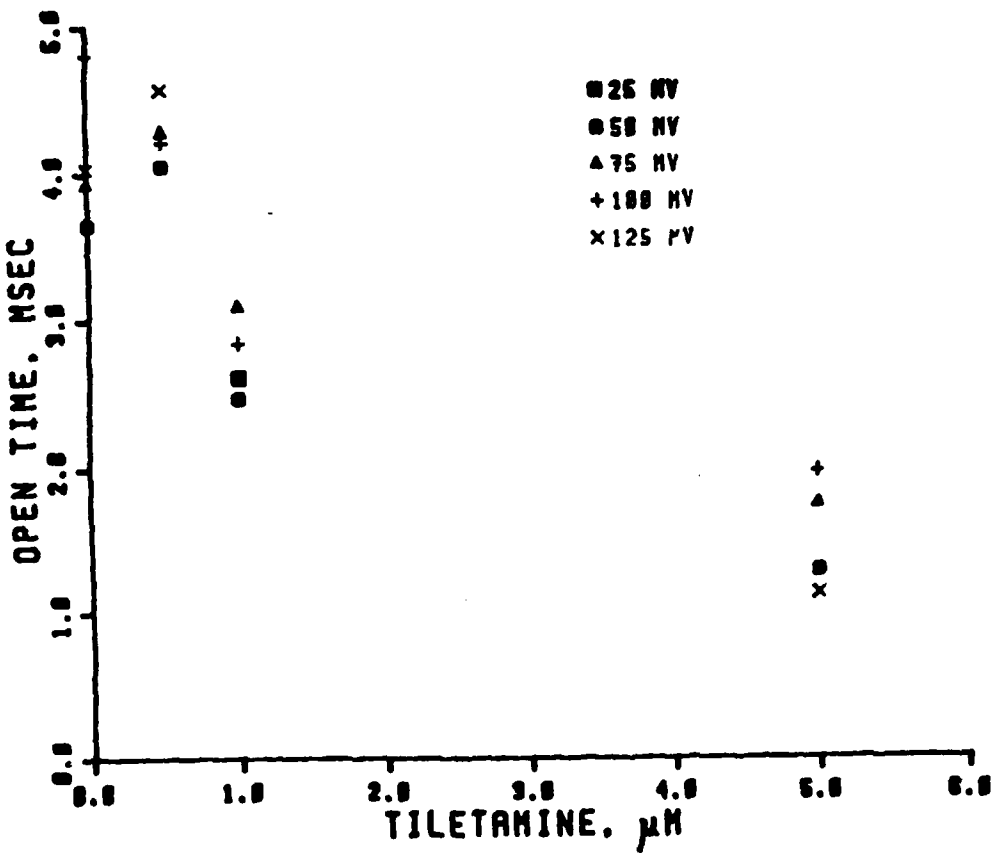


Figure 5. Effect of tiletamine on the mean channel open time for the acetylcholine receptor from rat myotubes. Acetylcholine (100 nM) and tiletamine were added to the pipette solution and the cell attached configuration was used. As indicated in the legend, the effect was tested at several holding potentials.

APPENDIX C
OVERALL STATUS OF THE REPORT

OVERALL STATUS OF THE PROJECT

This project has generated a significant amount of data concerning the structural aspects of binding sites on the nicotinic acetylcholine receptor. In particular, hydrophobicity requirements for binding to the site for noncompetitive blockers were elucidated as well as the stereospecificity of the opiate binding site. Two mechanisms of action for these compounds were defined: 1) An allosteric increase in channel lifetime and 2) a decrease in channel lifetime presumably due to a channel block mechanism.

Because of the large amount of data required for definitive structure-activity profiles, this project would benefit from additional data to be obtained using biochemical techniques, stopped-flow fluorescence measurements (to monitor AChR conformational changes), and single-channel recording. To this end, an unsolicited proposal was submitted to the U S Army Research Office which describes a plan for a continuation and extension of these studies.

END

FILMED

7-85

DTIC